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The α_1/β_1 and α_6/β_1 Integrin Heterodimers Mediate Cell Attachment to Distinct Sites on Laminin

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Abstract. This study was undertaken to determine the roles of individual α/β_1 integrin heterodimers in promoting cellular interactions with the different attachment-promoting domains of laminin (LN). To do this, antibodies to the integrin β_1 subunit or to specific integrin α subunits were tested for effects on cell attachment to LN, to elastase fragments E1-4 and E1, derived from the short arms and core of LN's cruciform structure, and to fragment E8 derived from the long arm of this structure. The human JAR choriocarcinoma cells used in this study attached to LN and to fragments E1 and E8. Attachment to E1-4 required a much higher substrate coating concentration, suggesting that it is a poor substrate for JAR cell attachment. The ability of cells to attach to different LN domains suggested the presence of more than one LN receptor. These multiple LN receptors were shown to be β_1 integrin heterodimers because antibodies to the integrin β_1 subunit inhibited attachment of JAR cells to LN and its three fragments. To identify the individual

integrin α/β_1 heterodimers that mediate interactions with these LN domains, mAbs specific for individual β_1 heterodimers in human cells were used to study JAR cell interactions with LN and its fragments. An anti- α_6/β_1 -specific mAb, GoH3, virtually eliminated cell attachment to E8 and partially inhibited attachment to E1 and intact LN. Thus the major α_6/β_1 attachment domain is present in fragment E8. An α_1/β_1 -specific mAb (S2G3) strongly inhibited cell attachment to collagen IV and partially inhibited JAR attachment to LN fragment E1. Thus, the α_1/β_1 heterodimer is a dual receptor for collagen IV and LN, interacting with LN at a site in fragment E1. In combination, the anti- α_1 - and anti- α_6 -specific antibodies completely inhibited JAR cell attachment to LN and fragment E1. Thus, the α_1/β_1 and α_6/β_1 integrin heterodimers each function as LN receptors and act together to mediate the interactions of human JAR choriocarcinoma cells with LN.

THE basement membrane glycoprotein laminin (LN)¹ is a large (M_r 850 kD) cruciform-shaped complex assembled from three different subunits (A, B1, and B2), which interacts with many diverse cell types (reviewed in Martin and Timpl, 1987). At least three proteolytic fragments of LN, E1, E1-4, and E8, have been shown to interact with cells in vitro (see Fig. 1). One of these, E8, which has an M_r of 280 kD (Paulsson et al., 1985) and contains most of the long arm of LN, including the heparin-binding fragment E3, has been shown to promote the attachment of many cell types (see Edgar et al., 1984; Aumailley et al., 1987; Goodman et al., 1987). A second large (M_r 630 kD) fragment, E1-4, derived from the short arms and core of LN (Ott et al., 1982), promotes the attachment of a distinct, but over-

lapping set of cells (Aumailley et al., 1987; Goodman et al., 1987). A smaller fragment E1 (M_r 200–260 kD; Ott et al., 1982), which contains a subset of the domains in E1-4, also promotes attachment of many cells, including some that do not adhere to fragment E1-4 and some which interact with intact LN only via sites in E8 (see Aumailley et al., 1987; Goodman et al., 1987; Nurcombe et al., 1989).

The differences between cells in recognition of the multiple attachment domains of LN are most easily explained by the existence of more than one LN receptor. Among receptor candidates are the integrins, implicated initially because antibodies to the integrin β_1 subunit were shown to inhibit strongly interactions of many cells with LN (see Horwitz et al., 1985; Bozyczko and Horwitz, 1986; Hall et al., 1987; Tomaselli et al., 1987, 1988). This suggested that heterodimers containing the integrin β_1 subunit and uncharacterized α subunits function as LN receptors. More recently, it has been possible to identify four integrin α subunits that associate with the integrin β_1 subunit to form LN receptors. One of these, α_3 , forms α_3/β_1 heterodimers that interact with

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1. *Abbreviations used in this paper:* Col IV, collagen IV; FN, fibronectin; LN, laminin.

several different ECM glycoproteins, including LN (Wayner and Carter, 1987; Wayner et al., 1988; Takada et al., 1988; Gehlsen et al., 1988, 1989). The second of these α subunits, α_6 , appears to form heterodimers that interact specifically with LN (Sonnenberg et al., 1988). A third distinct integrin β_1 heterodimer with an M_r 200-kD α subunit has been identified by LN affinity chromatography (Ignatius and Reichardt, 1988). An antibody to the α subunit of this heterodimer inhibits interactions of rat PC12 pheochromocytoma cells with LN and several collagens, but not fibronectin (FN) (Turner et al., 1989). Finally, an α_2/β_1 heterodimer has been reported to act as a LN receptor in some, but not all, cell types (Elices and Hemler, 1989; Languino et al., 1989).

In this study, the effects of antibodies to specific integrin heterodimers on cell interactions of the human choriocarcinoma cell line, JAR, with LN fragments were tested to identify which integrin receptors mediate cell attachment to specific domains of LN.

Materials and Methods

Materials

Costar 96-well flat-bottom Seroccluster plates (no. 3596) and 6-well plates were purchased from Costar Corp. (Cambridge, MA). FN was purchased from Collaborative Research (Lexington, MA). Preswollen diethyl aminoethyl cellulose (DE 52) was purchased from Whatman Biosystems Ltd. (Kent, UK). Protein A-Sepharose CL 4B, heparin Sepharose, and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). [3 H]N-Acetyl-D-glucosamine and En^hHance were obtained from New England Nuclear (Boston, MA). Ribi Adjuvant was purchased from Ribi Immunochemicals (Hamilton, MT). Unless otherwise indicated, all other chemicals, including BSA, and goat anti-rat IgG-agarose were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of LN, LN fragments and Polyclonal Antisera

LN and Col IV were purified from the murine Engelbreth-Holm-Swarm sarcoma by published procedures (Timpl et al., 1979; Kleinman et al., 1982). LN purity was assessed by SDS-PAGE. Western blotting with anticollagen IV antibodies showed collagen IV to be only a minor contaminant (not shown). The larger fragments of LN, E1-4 and E8, were prepared from LN digested with elastase (E0258, Sigma Chemical Co.) for 4 h at 37°C in 0.05 M ammonium bicarbonate (enzyme/substrate ratio, 1:100). Digestion was stopped by addition of PMSF and digests were immediately chromatographed on Bio-Gel A 1.5 m (2.5 × 120 cm) (Bio-Rad Laboratories, Richmond, CA) in 0.2 M ammonium bicarbonate at 4°C. This effectively separated fragments E1-4 and E8 from each other as well as from whole LN (Timpl et al., 1983; Goodman et al., 1987). Fragments E1-4 and E8 were purified further by chromatography on heparin Sepharose: E8 binds to heparin Sepharose, whereas E1-4 does not (Timpl et al., 1983). LN fragments E1 and E3 were purified from an overnight elastase digest of LN (enzyme/substrate ratio, 1:100) at 37°C as described (Ott et al., 1982). Polyclonal antisera to whole LN and to LN fragment E3 were raised in female White New Zealand rabbits by standard protocols. IgG was purified from rabbit sera by DEAE cellulose chromatography as previously described (Hudson and Hay, 1980). Affinity purified rabbit anti-collagen TIV was purchased from Dr. H. Furthmeyer (Stanford University, Palo Alto, CA).

LN fragments, summarized in Fig. 1, were identified by biological activity, SDS-PAGE profile, reactivity with the anti-E3 antibody and by structure as viewed by EM after rotary shadowing. Rotary shadowing was performed by dissolving LN and LN fragments in 0.1 M ammonium bicarbonate, 50% glycerol, then spraying onto freshly cleaved mica as previously described (Shotton et al., 1979; Engel et al., 1981).

Cells

JAR choriocarcinoma cells (Patillo, 1971) were cultured in DMEM with

0.4% glucose, 10% FBS and 50 μ g/ml gentamycin. Sp2/0 cells were cultured in H-Y medium (DME plus 10% NCTC 109, 10% FBS, 50 μ g gentamycin, 0.13% hypoxanthine, 0.04% thymidine; see Kennett et al., 1980). First-trimester human cytotrophoblasts were isolated from 8–12-wk placentas derived from elective abortions. The cytotrophoblasts were purified using sequential collagenase-hyaluronidase and trypsin treatment of placental villi according to Fisher et al. (1989). Cells were plated overnight on FN-coated 30-mm tissue culture plates in DMEM with 0.4% glucose, 10% FBS, and 50 μ g/ml gentamycin. Cells were scraped from the wells and rinsed twice with serum-free medium before injection into mice.

Cell Attachment Assay

Cell attachment to ECM ligands was measured using the assay described in Kramer et al. (1989b). Briefly, wells of 96-well plates were coated overnight with purified ECM ligands suspended in PBS at concentrations found to be optimal (5–10 μ g/ml LN, 5 μ g/ml FN, 0.5–5 μ g/ml Col IV, 2–10 μ g/ml E8, 100–200 μ g/ml E1-4, 1–10 μ g/ml E1). All wells were blocked with BSA for at least 2 h before use (0.2% BSA in PBS). BSA- and poly-D-lysine-coated wells served as negative and positive controls, respectively. Cells were harvested with 0.2% trypsin/2 mM EDTA in calcium-magnesium-free PBS (CMF-PBS), washed in serum free DME containing 0.4% soybean trypsin inhibitor and 0.2% BSA. Cells were plated at a density of 2×10^4 cells in 25 μ l in wells containing 75 μ l of hybridoma supernatant or purified immunoglobulin. After 1 h at 37°C, the plates were shaken on a Labline orbital shaker at 350 rpm for intervals sufficient to remove the cells from the BSA coated wells (10–30 s). Attached cells were fixed with 2% glutaraldehyde and photographed. Attachment levels were quantified either by cell counting or by quantifying trypan blue dye adsorption at OD₆₉₀, as previously described (Hall et al., 1987).

mAbs to Human Integrins

A Lewis rat was given two intraperitoneal injections 2 wk apart with 10^7 EDTA-harvested JAR choriocarcinoma cells, mixed 1:1 with Ribi adjuvant. 2 wk later, two additional intrasplenic injections were given 2 wk apart in the absence of adjuvant. A Balb/c mouse was given four bimonthly intraperitoneal injections of 5×10^6 first-trimester human cytotrophoblasts. 4 d after the last injection, each spleen was fused with Sp2/0 mouse plasmacytoma cells by the method of Kennett et al. (1980), as modified by Wheelock et al. (1987). Hybridoma supernatants were screened for their ability to inhibit JAR human choriocarcinoma cell attachment to FN, LN, or Col IV using the attachment assay described above. Two rat hybridoma supernatants were found that inhibited attachment to FN only (BIE5 and BIIG2), whereas two others inhibited attachment to LN, FN, and Col IV (AIIIB2 and BIE11). One mouse hybridoma supernatant inhibited attachment of JAR cells to Col IV only (S2G3). These hybridomas were cloned by limiting dilution. The rat antibodies were purified from culture supernatants by affinity chromatography using goat anti-rat agarose. The mouse supernatant, S2G3, an IgM, was concentrated 10-fold by precipitation with 50% saturated ammonium sulfate at 4°C. These antibodies were retested for attachment inhibitory activity on FN, LN and Col IV coated substrates before further use. The BIE5 rat mAb, which inhibits attachment to FN, LN, and Col IV; and S2G3 mouse mAb, which was identified by its inhibition of cell attachment to Col IV, were used in experiments reported here (Fig. 4).

The GoH3 mAb against the α_6 chain of the integrin β_1 family, was raised and characterized as described (Sonnenberg et al., 1986, 1988). An mAb against the α_1 chain of the β_1 family, TS2/7, was the kind gift of Dr. Martin Hemler (Dana Farber Cancer Institute, Boston, MA; Hemler et al., 1984). mAbs PIH5 and PIB5 that recognize α_2 and α_3 subunits of the β_1 family were kind gifts of Drs. Elizabeth Wayner and William Carter (Fred Hutchinson Cancer Center, Seattle; Wayner and Carter, 1987).

Immunoprecipitation

Attachment-inhibiting antibodies were characterized further by immunoprecipitation as follows. Cells were labeled with [3 H]N-acetyl-D-glucosamine (50 μ Ci/ml) over a 24-h period in low-glucose (0.1%), serum-containing DME. Cells were harvested with 2 mM EDTA in CMF-PBS-BSA, washed and lysed in 1 ml/ 10^7 cells of lysis buffer (10 mM Tris acetate buffer, pH 8.0 containing 0.5% NP-40, 100 mM NaCl, and 1 mM PMSF). Insoluble material was removed by centrifugation at 12,000 g for 10 min. Each ml of supernatant was precleared by two sequential 1-h incubations with 100 μ l packed, unconjugated Sepharose 4B beads. Aliquots of 250 μ l extract

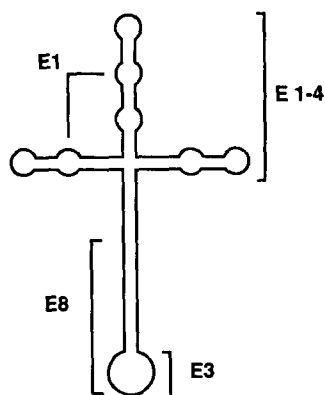


Figure 1. Laminin diagram. Diagram of intact LN delineating the location of the cell attachment domains discussed in this study.

were mixed two parts extract to one part mAb-containing or Sp2/0 supernatant. Immunoprecipitation was carried out as described in Kramer et al. (1989). Samples were fractionated by SDS-PAGE (Laemmli, 1970) and visualized by fluorography (Bonner and Laskey, 1974).

For sequential immunoprecipitations of extracts by the anti- α_1 mAb TS2/7, followed by S2G3 and AIIB2 mAbs, the precleared extract was incubated with anti- α_1 followed by two sets of goat anti-mouse beads. This cycle was repeated twice. The last round included three sets of goat anti-mouse beads to remove completely any remaining primary antibody. The depleted extract was then incubated with S2G3 or AIIB2 mAb and precipitated with goat anti-mouse IgG or goat anti-rat IgG beads. All samples were washed, eluted and analyzed by SDS-PAGE and fluorography as described above.

Results

1. Characterization of anti- β_1 Integrin mAbs (Table I)

An mAb to the human integrin β_1 subunit (AIIB2) was isolated and used to identify β_1 integrins on JAR cells. Several experiments indicated that this mAb recognized the β_1 subunit. First, SDS-PAGE analysis of immunoprecipitates of labeled JAR cell extracts showed that this antibody immunoprecipitated several integrin subunits. When analyzed in nonreducing conditions, broad bands of 110–120 and 145–

150 kD and a sharper band at 190 kD were present. When analyzed under reducing conditions, two bands were evident at 135–145 kD and 190 kD (Fig. 2, lanes A and E). This differential behavior on reduced and nonreduced gels is diagnostic of the integrin family of adhesion receptors (Knudsen et al., 1985; Giancotti et al., 1985; Pytela et al., 1986). Second, the antibody inhibited cell attachment to FN, Col IV and intact LN (Fig. 3), but not to vitronectin (not shown). This is a specificity similar to that exhibited by the antiavian integrin β_1 subunit-specific mAb, CSAT (Horwitz et al., 1985; Bozyczko and Horwitz, 1986; Hall et al., 1987; Neugebauer and Reichardt, unpublished experiments). These results indicate that the AIIB2 mAb binds the integrin β_1 subunit. This mAb is referred to subsequently as anti- β_1 .

To study the role of individual members of the β_1 integrin family in the attachment of the JAR human choriocarcinoma cell line to LN, attachment-inhibitory mAbs, raised against human choriocarcinoma and cytotrophoblast cells, were used. The mAbs were selected for their ability to disrupt cell-ECM protein interactions and were characterized further to determine their integrin heterodimer specificity. Two of the antibodies isolated in this way were used for the present study: BIE5, specific for the FN receptor, α_5/β_1 , and S2G3, specific for α_1/β_1 , a receptor for both LN and Col IV.

The BIE5 antibody appears to interact only with the FN-binding heterodimer α_5/β_1 . First, BIE5 inhibited JAR cell attachment to FN, but not to LN or Col IV (Fig. 3). It recognized two bands of 110–120 and 140–150 kD when analyzed by immunoprecipitation and SDS-PAGE under nonreducing conditions and a single band at 135–145 kD when analyzed under reducing conditions (Fig. 2, lanes C and F). Thus, the antibody appears to recognize only one heterodimer. This must be the α_5/β_1 heterodimer as this antibody recognizes a heterodimer with similar relative molecular mass subunits in the K562 cell line which expresses α_5/β_1 , but no other β_1 -containing integrins (Hemler, M., personal communication). This mAb is referred to henceforth as anti- α_5/β_1 .

The S2G3 mAb recognizes the integrin α_1/β_1 heterodi-

Table I. Antibodies Used in the Present Study

Antibody/ (*NR Ag Mr)	Antigen used/Made in	Alternative names	Inhibits attachment to
Anti- β_1 (110 kD)	JAR cells/Rat mAb	AIIB2 [‡]	FN, LN, Collagen
Anti- α_1/β_1 (190/110 kD)	Cytotrophoblasts Mouse mAb	S2G3 [‡]	Col, LN fragment E1
Anti- α_1/β_1 (190/110 kD)	T cells/Mouse mAb	Ts2/7, [§] anti-VLA1	No activity
Anti- α_2/β_1 (150/110 kD)	WI-38 human fibroblasts/ Mouse mAb	P1H5, anti-VLA2	Col
Anti- α_3/β_1 (150/110 kD)	WI-38 human fibroblasts/ Mouse mAb	P1B5, anti-VLA3	FN, LN, Collagen
Anti- α_5/β_1 (150/110 kD)	Jar cells/Rat mAb	BIE5 [‡]	FN
Anti- α_6/β_1 (135/110 kD)	Mammary tumor cells/ Rat mAb	GoH3 [†] anti-VLA6	LN, LN fragments E8, E1

* M_r of antigen analyzed by SDS-PAGE under nonreducing conditions.

[‡] Reported first in this study.

[§] Hemler et al. (1984), Takada et al. (1987).

^{||} Wayner and Carter (1987), Wayner et al. (1988).

[†] Sonnenberg et al. (1986, 1988).

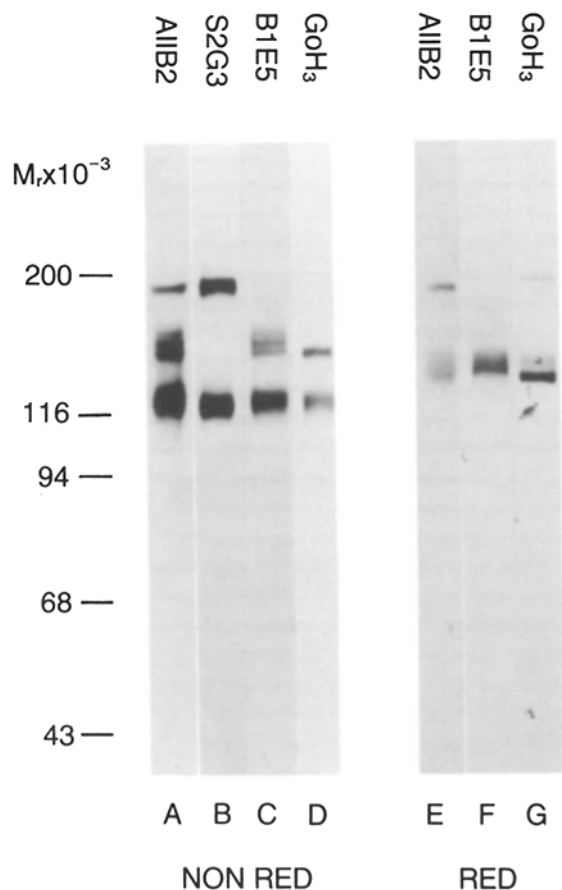


Figure 2. Analysis of antigens in JAR cells recognized by mAbs raised against members of the β_1 family of integrins. Extracts of [3 H]N-acetyl-D-glucosamine-labeled JAR cells were precipitated with mAbs and analyzed on 7.5% SDS-polyacrylamide gels under nonreducing (lanes A–D) or reducing (lanes E–G) conditions. Lanes A and E: anti- β_1 mAb (AIIB2); lane B, anti- α_1/β_1 mAb (S2G3); lanes C and F, anti- α_5/β_1 mAb (BIE5); lanes D and G, anti- α_6/β_1 mAb (GoH3).

mer. As shown in Fig. 3, the antibody inhibited JAR attachment to Col IV, had a small inhibitory effect on attachment to intact LN, but had no effect on attachment to FN. SDS-PAGE analysis of immunoprecipitates using S2G3 revealed two protein bands migrating with M_s of 110–120 and 190 kD under nonreducing conditions (Fig. 2, lane B). The 190-kD band did not change in apparent mobility upon electrophoresis under reducing conditions (compare 190-kD band in Fig. 2, lanes A and B with lane E). The protein bands recognized by the S2G3 mAb also comigrated with proteins immunoprecipitated with the anti-VLA-1 mAb, TS2/7, which defines α_1/β_1 (Hemler et al., 1984, 1987). Depletion of α_1/β_1 heterodimers by three rounds of sequential immunoprecipitation of JAR cell extracts with anti-VLA-1 mAb, removed the two proteins precipitated by the S2G3 mAb, but did not deplete the extract of other members of the β_1 family precipitated by anti- β_1 antibody (Fig. 4). Thus, the S2G3 antibody recognizes the integrin α_1/β_1 heterodimer and will be referred to as anti- α_1/β_1 .

The α_6 subunit-specific mAb GoH3, prepared using mouse mammary tumor cells as an immunogen (Sonnenberg

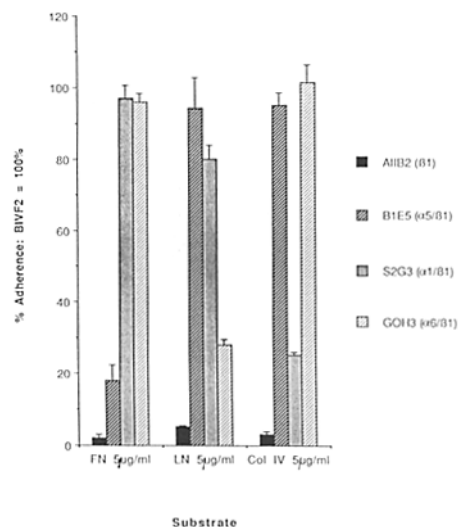


Figure 3. Effects of anti-integrin antibodies on JAR cell attachment to FN, LN, and Col IV. mAbs, generated using intact human JAR choriocarcinoma or primary cytotrophoblast cells as the immunogen (Materials and Methods), were selected for their ability to inhibit cell attachment to purified matrix protein substrates. Three mAbs generated in this manner recognized β_1 integrins: anti- β_1 (AIIB2; ■), anti- α_5/β_1 (BIE5; ■), and anti- α_1/β_1 (S2G3; ■) (see Table I for a complete list of antibodies). The anti- α_6 mAb (GoH3; □), generated using murine tumor cells as an immunogen, recognizes human α_6 integrin. The control mAb BIVF2, generated in the same fusion as the anti- β_1 mAbs, recognizes a 90-kD surface glycoprotein but does not effect cell-substrate interactions. Percent cell attachment, relative to positive (BIVF2) and negative (BSA) controls, is shown. Results from several experiments were pooled, the error bars denote SEM.

et al., 1986), also recognizes the α_6 subunit in human cells (Sonnenberg et al., 1987; Hemler et al., 1988). GoH3 inhibited JAR cell attachment to LN by ~70%, but did not affect attachment to either FN or Col IV (Fig. 3). In immunoprecipitation studies, GoH3 recognized proteins that migrated with M_s of 135 and 110–120 kD when analyzed under nonreducing conditions (Fig. 2, lane D). Under reducing conditions, the α_6 subunit migrated slightly faster than the β_1 subunit (Fig. 2, lane G). This antibody will be referred to as anti- α_6 .

The mAbs described above, as well as additional antibodies against the integrin α_2/β_1 and α_3/β_1 heterodimers (Wayner and Carter, 1987) were used in immunoprecipitation analyses to obtain a more complete picture of the variety of β_1 integrins expressed by JAR cells (see Table I). Anti- α_1/β_1 , anti- α_2/β_1 , anti- α_3/β_1 , anti- α_5/β_1 , anti- α_6 , and anti- β_1 antibodies were used to precipitate equal volumes of a [3 H]N-acetyl-D-glucosamine labeled JAR cell extract (Fig. 5). JAR cells expressed all of the integrins recognized by these antibodies except α_2/β_1 , which was undetectable by these procedures. The levels of α_3/β_1 also appeared to be low in these cells. These experiments are not sufficient to determine the absolute levels of the individual integrin heterodimers in JAR cells, because the efficiency with which each antibody precipitated its ligand may not have been equal. Comparative studies, however, using extracts from human placental fibroblasts showed relatively high levels of expres-

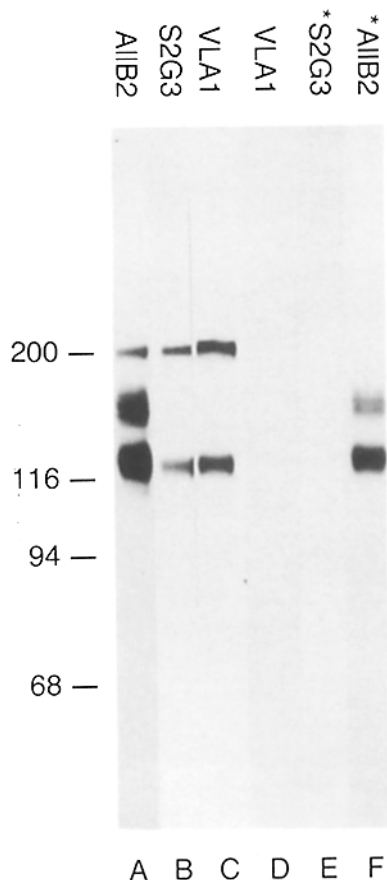


Figure 4. The S2G3 mAb recognizes the α_1/β_1 integrin heterodimer. Extracts of [^3H]N-acetyl-D-glucosamine-labeled JAR cells were precipitated sequentially by anti- α_1/β_1 (anti-VLA1) and either S2G3 or anti- β_1 (AIIIB2) mAbs as described in Materials and Methods and analyzed on 7.5% SDS-polyacrylamide gels under nonreducing conditions. A photograph of the fluorograph of the gel is shown. Lane A, anti- β_1 (AIIIB2) mAb precipitation of an undepleted extract; lane B, anti- α_1/β_1 (S2G3) mAb precipitation of an undepleted extract; lane C, anti- α_1/β_1 (anti-VLA 1) first precipitation; lane D, anti- α_1/β_1 (anti-VLA 1), third precipitation; lane E, anti- α_1/β_1 (S2G3) precipitation of anti- α_1/β_1 (anti-VLA1) depleted extract; lane F, anti- β_1 mAb (AIIIB2) precipitation of anti- α_1/β_1 (anti-VLA1)/depleted extract.

sion of α_2/β_1 and α_3/β_1 , but a low level of α_6/β_1 (not shown). Thus, the antibodies to α_2/β_1 and α_3/β_1 are capable of precipitating their respective antigens effectively from whole cell extracts. Consequently, the α_2 and α_3 subunits appear to be expressed at low levels in JAR cells compared with the α_1 , α_5 , and α_6 subunits.

II. Attachment of JAR Cells to LN Fragments Is Inhibited by an Anti- β_1 Integrin Antibody

LN fragments E1-4, E1 and E8, which have been shown previously to support cell adhesion, were prepared as described in Materials and Methods (see Fig. 1 for structures). The attachment of the human JAR choriocarcinoma cell line to LN and LN fragments was measured using cell attachment assays. JAR cells, a tumor cell line of epithelial origin attached to whole LN as well as to fragments E1-4, E1, and E8. Con-

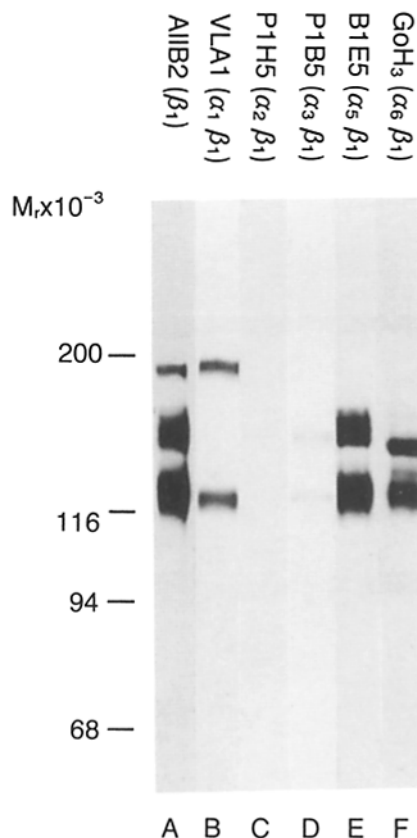


Figure 5. JAR cells express several members of the β_1 family. Equal aliquots of an extract of [^3H]N-acetyl-D-glucosamine-labeled JAR cells were precipitated with the antibodies indicated, and analyzed as in Fig. 4. JAR cells express relatively high levels α_1/β_1 , α_5/β_1 and α_6/β_1 and low levels of α_3/β_1 . α_2/β_1 is not detectable in JAR cells under the conditions used. Identical analysis of human placental fibroblast cell extracts shows relatively high levels of α_1/β_1 , α_2/β_1 , α_3/β_1 , and α_5/β_1 and low levels of α_6/β_1 (not shown), indicating that all the antibodies used are capable of precipitating their respective antigens under these conditions.

centration curves (not shown) indicated that maximal attachment of JAR cells to LN and its fragments occurred as follows; LN, 2 $\mu\text{g}/\text{ml}$; E8, 1–2 $\mu\text{g}/\text{ml}$; E1, 1–2 $\mu\text{g}/\text{ml}$; E1-4, 100–200 $\mu\text{g}/\text{ml}$. Thus, E1-4 is a much less effective attachment substrate than whole LN, E1 or E8. Antibodies to fragment E3 inhibited cell attachment to fragment E8 completely, and reduced slightly the attachment to whole LN, but did not inhibit cellular interactions with either fragment E1-4 or E1 (Fig. 6). These results suggested that JAR cells attach to at least two sites on LN, one present in fragment E8 and the other in fragments E1-4 and E1.

To examine the functions of β_1 integrin heterodimers in JAR cell attachment to LN, JAR cells were plated on LN, LN fragments E1-4, E1, and E8, in the presence of either anti- β_1 or an irrelevant mAb that binds to JAR cells but has no adhesion disrupting activity (BIVF2). As shown in Fig. 7, anti- β_1 inhibited JAR cell attachment to LN and to all three fragments. It had no effect on attachment to poly-D-lysine (not shown).

These results indicate that the β_1 integrin family of adhe-

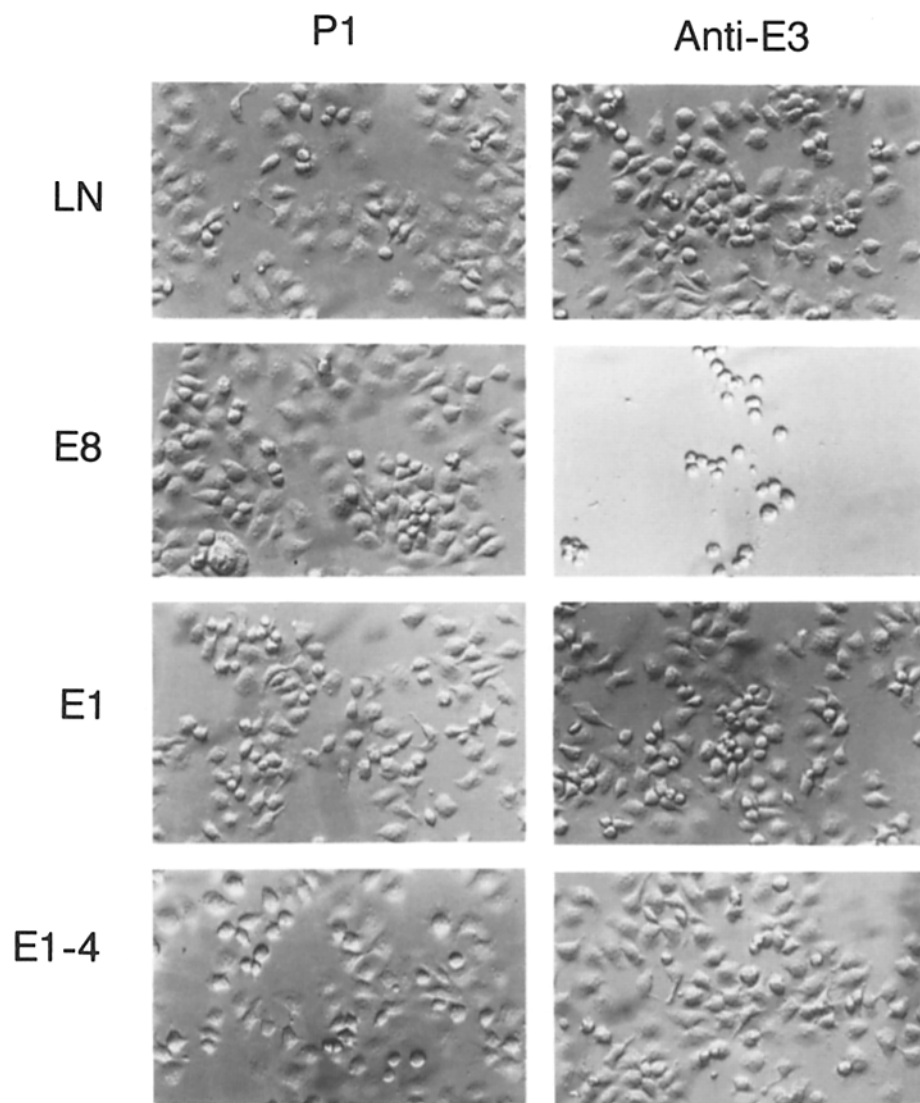


Figure 6. Anti-E3 antibody inhibits JAR cell attachment to E8, but not to the E1 or E1-4 fragments of laminin. The figure shows photomicrographs of JAR choriocarcinoma cells plated on LN and LN fragments E1-4, E1 and E8, in the presence of anti-E3 antibody (100 μ g/ml) or preimmune IgG control (100 μ g/ml) mAb. Cells were treated as for the attachment assay (see Materials and Methods) and photographed after unattached cells were removed. Bar, 100 μ m.

sion receptors plays a dominant role in the JAR cell attachment to these three attachment-promoting domains of LN.

III. Integrin α_1/β_1 and α_6/β_1 Heterodimers Mediate JAR Cell Attachment to the E1 and E8 Attachment-promoting Domains of LN

To determine the role of individual integrin heterodimers in JAR cell binding to LN, the mAbs recognizing individual heterodimers were tested for their effects on the attachment of JAR cells to LN and to fragments E1 and E8. E1-4 was not used in these experiments due to the very high substrate coating concentrations required for optimal cell attachment. As shown in Fig. 7, antiintegrin α_1/β_1 blocked JAR cell attachment to E1 by 60–70%, but did not have significant effects on JAR cell attachment to E8. Antiintegrin α_1/β_1 inhibited interaction with whole LN \sim 20% (Fig. 7). Thus, the integrin α_1/β_1 receptor recognized by the S2G3 mAb interacts with sites on the E1 fragment of LN. In contrast, anti- α_6/β_1 antibody inhibited JAR cell attachment to fragment E8 by >90%. This indicates that the principal interaction of JAR cells with fragment E8 is mediated by the integrin

α_6/β_1 receptor. Anti- α_6/β_1 antibody also blocked attachment of JAR cells to intact LN and to fragment E1 by \sim 50%. These results suggest that the integrin α_6/β_1 heterodimer interacts with a second distinct site in LN, which is in the E1 domain. They also indicate that the principal JAR attachment site in intact LN is in the E8 fragment. As expected, a mAb specific for integrin α_5/β_1 , the fibronectin receptor, had no inhibitory effects on JAR cell attachment to LN or any of its fragments.

Since the effects of α_1/β_1 -specific and α_6/β_1 -specific mAbs on the attachment of JAR cells to fragment E1 were partial, a mixture of the two antibodies was tested. Results in Fig. 7 show that such a mixture was more effective in inhibiting JAR attachment to intact LN and to fragment E1 than either antibody alone. This antibody combination inhibited JAR attachment to LN by 85% and to E1 by 92% (Fig. 7). This same combination of antibodies did not inhibit cell attachment to FN, nor did anti- α_6/β_1 antibody enhance the inhibitory effect of anti- α_1/β_1 antibody alone on cell attachment to Col IV. Finally, the anti- α_5/β_1 mAb, which recognizes a FN receptor, did not enhance the effects of anti- α_1/β_1 on cell attachment to fragment E8 or to LN. This indicates that anti-

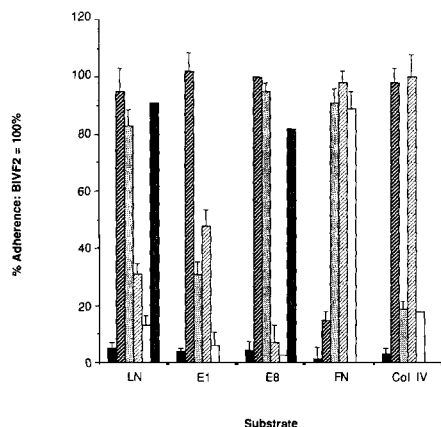


Figure 7. Monoclonal antibodies recognizing α_1/β_1 , and α_6/β_1 integrins inhibit JAR attachment to LN, E1-4, and E8. JAR choriocarcinoma cell attachment to LN (5 $\mu\text{g/ml}$), LN fragments E1-4 (140 $\mu\text{g/ml}$), E8 (5 $\mu\text{g/ml}$), FN (5 $\mu\text{g/ml}$), and collagen IV (5 $\mu\text{g/ml}$) was measured in the presence of anti-integrin antibodies singly and in combination, as indicated in the figure. Percent cell attachment, relative to positive (BIVF2) and negative (BSA) controls, is shown. Data from several different experiments were pooled. Error bars denote SEM. ■, A1IB2 (β_1); ▨, B1E5 (5/ β_1); ▩, S2G3 (1/ β_1); □, GoH3 (6/ β_1); □, S2G3 + GoH3; ▨, S2G3 + B1E5.

bodies specific for closely related integrin heterodimers do not have nonspecific cooperative effects.

Together, the data presented here suggest that there are two β_1 integrin receptors for LN on JAR cells, α_1/β_1 and α_6/β_1 . The α_1/β_1 complex recognizes a domain present in E1 whereas, integrin α_6/β_1 recognizes fragment E8. The observation that anti- α_6/β_1 also partially inhibits JAR cell attachment to E1 suggests that α_6/β_1 may also recognize a distinct site in E1. The ability of a mixture of the two antibodies specific for these receptors to almost completely inhibit attachment of JAR cells to LN suggests that integrin α_1/β_1 and α_6/β_1 heterodimers mediate the major interactions of JAR cells with this ECM glycoprotein.

Discussion

Results in this paper indicate that LN contains at least two distinct cell attachment-promoting domains. Results also indicate that integrin β_1 subunit-containing heterodimers are used by JAR cells as the dominant receptors for interacting with these attachment-promoting domains of LN. We provide strong evidence that two individual integrin heterodimers, α_6/β_1 and α_1/β_1 , function as receptors for intact LN and our data identify the domains on LN with which these receptors interact. Integrin α_1/β_1 is shown to be a dual receptor for collagen IV and for intact LN, with which it interacts in the E1 fragment. Integrin α_6/β_1 interacts primarily with LN using a site in fragment E8; but may also interact with a second site in fragment E1. The results suggest that the major JAR cell recognition site in intact LN is in the E8 fragment. However, since anti- α_1/β_1 consistently inhibits JAR cell attachment to intact LN by a small amount (20%), and complements the effects of anti- α_6 on intact LN, the site(s) in the E1 region recognized by α_1/β_1 is at least partially accessible in intact LN. Thus, integrin α_1/β_1 and

α_6/β_1 heterodimers appear to act in concert to mediate the interactions of JAR cells with LN.

Previous work from several groups has provided evidence for attachment-promoting domains in LN fragments E8, E1-4, and E1 (Edgar et al., 1984; Paulsson et al., 1985; Engvall et al., 1986; Aumailley et al., 1987; Goodman et al., 1987; Dillner et al., 1988; Terranova et al., 1983; Timpl et al., 1983). Results in this paper provide further evidence for the existence of multiple cell attachment domains in LN. JAR cells were shown to interact with fragment E8, fragment E1 and, to a lesser extent, E1-4, implying that there are at least two separate attachment sites in intact LN.

Antibodies to the integrin β_1 subunit have been previously shown to inhibit interactions of many cell types to LN and this has suggested strongly that integrin β_1 subunit-containing heterodimers include receptors for LN (see Horwitz et al., 1985). Results in this paper extend this work by showing that an anti-integrin β_1 antibody inhibits interaction of human cells to the three attachment-promoting LN fragments described above (Fig. 6). This antibody also inhibited cellular interaction with FN and Col IV. Since some cell types have been shown to interact only with certain of these LN fragments (E1-4, E1 and E8) (Edgar et al., 1984; Goodman et al., 1987; Aumailley et al., 1987), it seemed likely that there are multiple, distinct β_1 receptors mediating cellular interaction with LN.

Recent work, when combined with results in this paper, indicates that four distinct integrin α subunits, when complexed with the integrin β_1 subunit, form receptors that bind LN. In this paper, we have isolated the mAb S2G3 and have provided explicit evidence that it binds the human integrin α_1/β_1 heterodimer (Fig. 4). Using this antibody, it has been possible to inhibit cellular interactions with both LN and Col IV (Figs. 3 and 7), providing clear evidence that in JAR cells the integrin α_1/β_1 heterodimer functions as a receptor for both of these ECM glycoproteins. Using fragments of LN, we have shown that this heterodimer interacts with LN fragment E1 (Fig. 7). It seems likely that this is the same site that the α_1/β_1 heterodimer uses to interact with intact LN, but this has not been demonstrated explicitly, and cannot be assumed, as it has been shown that "cryptic" sites for binding of some cell types are exposed by proteolysis (Aumailley et al., 1987). In previous work, a rat integrin β_1 subunit-containing heterodimer with an M_r 200-kD α subunit has been shown to exhibit direct, divalent cation-dependent binding to LN (Ignatius and Reichardt, 1988). A mAb (3A3) that appears to be specific for this integrin α subunit in the rat inhibits interactions of the rat pheochromocytoma cell line, PC12, to LN and to collagens I and IV (Turner et al., 1989). The size and binding specificity of the integrin heterodimer identified with this antibody suggests strongly that this α subunit is the rat homologue of human integrin α_1 . More recently, Kramer and Marks have shown that VLA-1 (α_1/β_1) binds to immobilized collagen I and IV (1989).

Evidence that integrin α_6/β_1 heterodimers mediate cell attachment to LN has been obtained with an α_6 -specific mAb, GoH3, which inhibits platelet attachment to LN, but not to FN, collagens I and III, or fibrinogen (Sonnenberg et al., 1988). In the present study, this integrin α_6 -specific antibody inhibited interactions of JAR cells with intact LN by $\sim 70\%$. The same antibody virtually eliminated interactions of these cells with LN fragment E8 and strongly reduced in-

teractions with LN fragment E1 (Fig. 7). These results imply that the integrin α_6/β_1 heterodimer interacts with at least two separate sites in LN, one in fragment E8 and one in fragment E1. The partial inhibitory effects of antiintegrin α_6/β_1 on cell attachment to E1 raises the possibility that E1 is contaminated with E8 or a smaller fragment of E8. Though anti-E3 antibody did not inhibit JAR cell attachment to fragment E1, the possibility remains that E1 is contaminated with a portion of E8 missing the anti-E3 domain. E8-like fragments of LN lacking E3 have been purified and shown to have cell attachment activity (reviewed in Engel et al., 1990). If such an E8-like fragment was contaminating our E1 preparation, JAR cell attachment to it would not be inhibited by anti-E3 antibody. Thus, the failure of the anti-E3 antibody to inhibit JAR cell attachment to E1 does not eliminate the possibility of contamination by these smaller E8-like fragments. Cell attachment activity in E8, but not E1, however, is very thermolabile (Goodman et al., 1987). The E1 preparation used in this study retained ~60% of its cell attachment-promoting activity after heating and was still subject to inhibition by anti- α_1 and anti- α_6 mAbs (data not shown). These results suggest strongly that the interactions between our E1 preparations and α_1/β_1 integrin are not due to contamination by E8 or fragments derived from E8. These results imply as well, that α_6/β_1 recognizes a heat stable site in E1 that is distinct from the site it recognizes in E8. The structural similarity or physical proximity of these two sites was not addressed specifically in these experiments and the issue of α_6 interaction with E1 requires further study (see also Sonnenberg, 1990).

Two and possibly three additional α subunits, α_2 (Elices et al., 1989; Languino et al., 1989), α_3 (Gehlsen et al., 1988, 1989) and a putative α_7 (Kramer et al., 1989a), also form heterodimers that interact with LN in at least some cells. The mAb PIB5 to the α_3/β_1 heterodimer (Wayner and Carter, 1987; Wayner et al., 1988) has been shown to inhibit attachment of human HT 1080 fibrosarcoma cells to LN, FN and collagens I and VI. In this study, the α_3/β_1 subunit is expressed at low levels in JAR cells (Fig. 5). The anti- α_3/β_1 did not affect JAR cell attachment to LN, LN fragments or collagen IV when used alone, and did not complement the effects of anti- α_1/β_1 or anti- α_6 on JAR cell attachment to LN and collagen IV (not shown). In other studies, the α_3/β_1 from rat glioma cells has been purified and shown to exhibit direct, divalent cation-dependent binding to LN and FN but not collagen (Gehlsen et al., 1988), whereas a similar preparation from human MG 63 cells binds all three ligands (Gehlsen et al., 1989). In assays measuring binding of purified α_3/β_1 heterodimers to LN, the only anti-LN mAbs that inhibited binding, recognize LN epitopes localized to the E8 fragment (Gehlsen et al., 1989), implying that this integrin heterodimer binds exclusively to a domain in the long arm of LN. The data in this paper, combined with those of Gehlsen et al. (1988, 1989), therefore, indicate that two different β_1 heterodimers (α_6/β_1 and α_3/β_1) recognize the E8 fragment.

More recently, α_2/β_1 has been shown to act as a LN receptor in melanoma cells (Elices, 1989) and in endothelial cells (Languino et al., 1989). Anti-integrin α_2/β_1 did not have an effect on JAR attachment to either LN or Col IV (not shown), again, probably due to the paucity of α_2/β_1 (Fig. 5).

Using the anti- α_1/β_1 and α_6/β_1 antibodies in combination,

it was possible to demonstrate the cooperative nature of receptor-LN interactions. The anti- α_1/β_1 and anti- α_6/β_1 antibodies together inhibited almost completely JAR cell interaction with both intact LN and with fragment E1 (Fig. 7). The addition of antibodies reactive with other integrin heterodimers did not enhance the inhibitory effect of either anti- α_1/β_1 or anti- α_6/β_1 . Thus, the integrin α_1/β_1 and α_6/β_1 heterodimers function independently and cooperatively to promote JAR cell attachment to LN.

The combined results from this and other recent reports indicate that it is increasingly difficult to make predictions about the integrin α subunit repertoires of cells that interact with LN. At least four heterodimers of the β_1 family have been shown to interact with LN. Several additional factors besides the presence of the α subunit are also likely to affect substrate specificity. For example, in some cells, the α_6 subunit has also been found associated with a novel β subunit, β_4 (Sonnenberg et al., 1987; Kajiji et al., 1989). Thus, differences in β subunit associations may modify the ligand specificity of α_6 -containing heterodimers as is the situation with the vitronectin receptor α subunit (Cheresh et al., 1989). Additional factors capable in principle of modifying integrin function include posttranslational modifications and receptor interactions with gangliosides, the cytoskeleton and accessory proteins (see Horwitz et al., 1986). Such mechanisms may explain cell type-specific differences in the ligand recognition properties of a particular integrin heterodimer (Gehlsen et al., 1989; Languino et al., 1989).

The above discussion points to convincing evidence for several distinct integrin α/β_1 heterodimers that bind to two or more attachment sites in LN, but the biological significance of these multiple receptors and attachment sites is not yet clear. One possibility is that individual receptors distinguish between the different forms of LN that occur in vivo. Variant forms of LN might arise from the differential synthesis or localization of the LN B1, B2 and A chains, which has been observed during development (Cooper and MacQueen, 1983; Klein et al., 1988). A homologue of the laminin B1 subunit, named S-laminin, has recently been described and shown to have a restricted temporal and anatomical distribution (Hunter et al., 1989). Additional homologues of the A, B1, and B2 chains may also exist (e.g., Aratani and Kitawa, 1988). Each of these may be recognized by only some of the existing LN receptors. In addition, LN forms complexes with many other ECM components, such as entactin, heparan sulfate proteoglycans, and collagen IV (reviewed in Martin and Timpl, 1987). Individual LN cell attachment sites may be shielded or exposed by these associated molecules. Finally, different LN isoforms do appear to have different functions. Development of kidney epithelial polarity, for example, has been shown to require expression of the LN A chain (Klein et al., 1988). Thus, differentiation of specific cells may well require function of a specific integrin heterodimer.

In conclusion, there is considerable heterogeneity in LN-containing structures. This is likely to be the case for other large multifunctional ECM ligands as well. Therefore, what appears to be a redundancy in integrin receptors may in fact be necessary to allow cells to interact with ECM ligands in some environmental contexts but not in others. Specific receptor-ECM interactions may also result in the transmission of distinct signals to the cell interior (Werb et al., 1989).

Thus, the heterogeneity in receptor-ligand interactions documented in this and other studies may constitute a basis for cells to sense and to communicate to the cell interior changes in their extracellular environment.

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